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# APPLICATION FOR U.S. PATENT

**TITLE:** RECOMBINATION ASSEMBLY OF LARGE DNA FRAGMENTS

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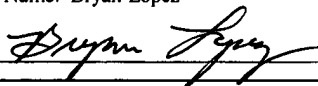
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## RECOMBINATION ASSEMBLY OF LARGE DNA FRAGMENTS

### PRIOR RELATED APPLICATIONS

[1] This patent application claims priority to U.S. Provisional Application Serial No. 60/422,748, filed on October 31, 2002, the disclosure of which is incorporated by reference  
5 in its entirety herein.

### FEDERALLY SPONSORED RESEARCH STATEMENT

[2] Not applicable.

### REFERENCE TO MICROFICHE APPENDIX

[3] Not applicable.

### FIELD OF THE INVENTION

[4] A process for recombination assembly of a series of cloned DNA fragments into a large ordered arrangement.

### BACKGROUND OF THE INVENTION

[5] With the structural analysis of DNA proceeding at a rapid pace based on the  
15 advances in DNA sequencing techniques and encouraged by potential applications of information from completely sequenced genomes of important organisms, a new chemical horizon is the synthesis of large DNA structures. This endeavor follows the trend of past chemical work on molecules of all types; first, determination of the structure, then synthesis of the structure for confirmation and producing novel structures and analogs to investigate their  
20 properties. A number of DNA analogs have been synthesized for special purposes (peptide backbone DNA, DNA with modified phosphoryl groups) and special sequence segments have been designed to interact with double helical DNA. In the case of microbial genomes, there has been discussion of preparing a minimal genome. However, general methods for constructing large precisely designed DNA segments have not been developed.

[6] Presently, commercially available oligonucleotide synthesis can routinely produce molecules on the order of a hundred nucleotides, and through PCR amplification of known segments of a genome, defined fragments of up to 40 kilobase pairs can be prepared. Through cleavage with specific restriction enzymes and joining by ligation, designed DNA molecules (e.g., large vectors) have been made. However, this method becomes complicated as larger fragments with more restriction sites are used and each molecule to be made must have its unique route of synthesis depending on its particular arrangement of restriction endonuclease sites. The protection of certain sites by specific methylases and the recent discovery of several very rare cleaving endonucleases have extended the range of manipulations available from this basic approach.

[7] Cloning techniques have been used to isolate and propagate large fragments on special vectors (BAC, YACs) and homologous DNA recombination has allowed the reconstruction of known chromosomal regions of over a hundred kilobase pairs. Improved systems for direct recombination have made functional studies of genes easier through “gene knockout technology” [4]. However, the defined assembly of a novel DNA sequence of large size has not been carried out. In order to produce large designed segments that are composed of DNA sections not normally together (or not even from the same organism), new methodologies need to be developed.

[8] Potential uses for these techniques are in areas such as the analysis of function of large interrupted coding regions that exist in many human genes, and in the construction of gene sets involved in complex metabolic processes [2]. These techniques could allow for more extensive genetic reprogramming of microbes for optimal production processes (metabolic engineering) and proposals for large scale “editing” of known genomes have been made based on engineering optimization considerations [3]. Methods for the generation of such DNA would allow the formation of optimized strains for industry and provides a way to explore global structural effects in the function of microbial genomes.

## SUMMARY OF THE INVENTION

[9] A general synthetic approach for the formation of designed unique DNA molecules of a size of hundreds of kilobase pairs has been developed. In general, the technique uses site specific recombinases to insert a vector containing a fragment of interest into a specific location on DNA in the cell. The chromosome or plasmid has been manipulated to contain a single recombinase site and a single excision site. These sites can be incorporated in to the cell DNA by any means known in the art, for example homologous recombination at the lac operon with the appropriate vectors.

[10] Next, the unnecessary vector sequences are excised by a first excisionase, thus bringing the fragment of interest adjacent to a prior inserted fragment and leaving the initial site specific recombinase site intact. This is possible because the vector also contains a site specific excision site (e.g. a recombinase site in the reverse orientation to that found in the recipient DNA).

[11] This process is repeated with a second vector that contains the same site specific recombinase site and a single excision site from a second excision enzyme. The first vector also included the second excision site in the opposite orientation. Thus, incorporation of the second vector into the chromosome now allows the intervening vector sequences to again be removed with the second excisionase.

[12] Using alternating excisionases, an unlimited number of fragments can be aligned adjacent in the chromosome, BAC or YAC DNA. We have exemplified the method using alternating excisionases for simplicity, but of course, three or more excisionases may be used and this is explicitly stated to be an equivalent of using two excisionases. When fragment assembly is complete, a final excisionase can be used to excise and circularize the assembled fragment provided the final excisionase sites were appropriately placed in the chromosome (or DNA element in the cell) and the final vector.

[13] The cre-lox system is the most commonly used site specific recombination system, but the art teaches a very large variety of site specific recombinases that are too numerous to name. Many of these recombinases can be employed as an "excisionase" in the

context of this invention, merely by placing the sites in the opposite orientation (See fig. 1). Suitable site specific recombinases include FRT, hix/hin, FlpR, xerD, shufflon, SSV1 integrase, and members of the Tn3 family, including the IS6 family of recombinases. Features of several site specific recombinases are mentioned below.

5           [14]   Several site specific recombinases have been used for excision of fragments from chromosomal DNA in *E. coli* or other organisms. Applications have included the removal of antibiotic resistance elements after a genetic manipulation. The lambda int/xis system has been used in *E. coli* [5] and mammalian cells [6]. The FRT/FLP system from yeast has been used in *E. coli* [7] [2], *Vibrio* [8], and has shown capability for deletion of large segments of 100 kb in *E.*  
10 *coli* [9]. A genetically improved flp recombinase has also been utilized [10]. A similar size deletion was also made in *E. coli* with the cre/lox system [11] and in *Pseudomonas* by the site-specific resolution system of Tn1722 [12].

          [15]   The Cre/lox system is one of the most widely used systems for deletions in plant [13], yeast [14, 15], *E. coli* [16], and mammalian systems [17]. Another group of site specific  
15 recombinases capable of precise deletion, or excision of the transposon are those derived from the conjugative transposons. The deletion of the transposon from insertion sites placed in *E. coli* has been studied with the transposons Tn916 and Tn1545. Analysis of the lambda int-like enzymes [18, 19], sites [20] and *in vitro* mechanism [21-23] of Tn 916 or Tn1545 [24, 25] has shown how these can be used to insert and excise DNA contained within the ends of the  
20 transposon.

          [16]   Recombination of a plasmid bearing a site for action of a recombinase into chromosome of a host cell has been used for manipulation in *E. coli* via the lambda system [6, 26] the Flp Frt system [27], or the cre/lox system [28]. The cre lox system has been used to bring large fragments (230 kb) into plant chromosomes [29]. Site specific recombinases have  
25 been used to manipulate the chromosomes of *Drosophila* (Flp-frt system) [30] and a concerted effort has shown the application of a number of systems in mammalian cells; lambda int system [31], phage phiC31 integrase [32], cre/lox [33, 34], [35], [36], streptococcal plasmid beta recombinase [37], and phage R4 integrase [38].

[17] Another group of recombinases capable of site specific deletion reactions include some of those usually involved in inversion of segments of the genome (invertase family) or resolution of dimeric structures after replication or cointegrate formation (resolvase family). In the Hin/hix inversion system [39] [40], inversion is preferred over deletion [41]. These  
 5 recombinases are stimulated by binding of Fis at a nearby site. Mutants of Hin which can catalyze deletions at high frequency have been described [42]. These mutations are in the E-helix as with Gin and other related recombinases that have lost enhancement or specificity functions.[43]

[18] In the case of Tn3 resolvase [44] [45], a mutant resolvase, with a D102Y mutation  
 10 [46] can act on inverted res sites as well as acting on a pair of sites one of which has a full res site and the other which contains only binding site I, and not binding site II or III of the full res site.

[19] Recently the beta recombinase of pSM19035 has been studied and the minimal recognized recombination site has been defined [47], a 90 bp site with two binding site elements  
 15 (site I and II) with recombination taking place in site I. [48]. Resolution also occurs on inverted sites in supercoiled plasmids [49], so an excision event can also be catalyzed.

[20] We have exemplified the system using FRT as the site specific recombinase, hin/hix as the first excisionase, TnE (L/R)/Tn excisionase as the second excisionase, and cre-lox as the final excisionase. The method is illustrated in Fig. 2A and 2B.

## 20 BRIEF DESCRIPTION OF THE DRAWINGS

[21] Fig. 1. Kinds of Site Specific Recombinases

[22] Fig. 2A and 2B. Integration and Excision Scheme.

## DESCRIPTION OF EMBODIMENTS OF THE INVENTION

[23] As used herein, all recombinases and excisionases are "site specific" enzymes.  
 25 "Chromosomal element" is a chromosome or a synthetic chromosome-like element (such as a BAC or YAC). "Replicon" is any nucleic acid that can be maintained by a cell.

[24] Beyond a certain size it becomes difficult to manipulate DNA *in vitro* and introduce it into cells, so an attractive approach is to assemble the larger segments in an already compacted form *inside* the cell. This invention is directed to a general method for precise DNA fragment assembly of large constructs using removable carrier DNA elements to bring the  
5 desired ends together. The method utilizes two separate recombination enzymes to join DNA fragments attached to the ends of the designed recombination cassette, specifically the systems for site specific recombination to Tn1545 [24, 25] or hix [41, 42]. The required recombinases are engineered to be present in the cell (e.g., on one or more plasmids) and their expression can be induced at the appropriate time in the assembly procedure.

[25] The procedure can be preformed *in vitro*, but the advantage of performing the assembly inside an intact cell is the ease of generating a very large DNA without incurring the risk of shearing the DNA. Nonetheless, smaller fragments (on the order of BAC or YAC size) can successfully be assembled *in vitro*. When the procedure is performed *in vivo*, the upper limit on size may be 100, 150, 200, 250, 500, 1,000, 1,500, 2,000 for *Streptococcus*, or  
15 *Haemophilus*, 3,000, 5,000 kb for *Salmonella* for *E. coli*, and 10,000 to 30,000 kb for yeast.

[26] The shuttle plasmid for delivery for DNA fragments bears an appropriately oriented recombination site (Tn end or hix site) and an FRT site allowing the plasmid to integrate at the unique chromosomal FRT site [27]. The receiving chromosomal construct for assembling fragments bears the right Tn end (or hix site) adjacent to a FRT site [5]. Assembling of DNA  
20 fragments into one segment is performed sequentially in the chromosome. DNA fragments subcloned into the shuttle plasmid between the Tn ends and the hix site are delivered into the chromosome via recombination between the chromosomal and plasmid FRT sites.

[27] The second recombination event is then initiated between active Tn ends (or hix sites) and excises a vector part of the shuttle plasmid leaving the delivered DNA fragment with  
25 its adjacent Tn end (or hix site) and one FRT site joined to the previously assembled DNA segment in the chromosome. Thus the DNA segments formerly attached to the end of the "carrier" modules are now joined together in a defined fashion after removal of the intervening functional carrier. The system is designed so successive rounds can be conducted using appropriate vectors, selection and recombinases (either Tn or hix).

## EXAMPLE 1.

[28] Individual steps of the process have been performed with success in a whole cell *E. coli* K-12 derived strains transformed with plasmids bearing the site specific recombination sites, and some combinations have been successfully performed. However, the whole assembly  
 5 has not yet been completed. To date, the following steps have been performed: 1) integration of *frt* containing plasmid into the chromosome using expression of FLP; 2) excision of Tn1545 bounded segment from a construct with Tn1545 *int/xis*; and 3) excision of *hix* bounded segment with *hin*.

[29] The following vectors were employed in the test reactions:

10 **Table 1: Vectors, Induction system and Citations**

Vector	Induction System	Citation
pKH66	p-tac promoter controlled <i>hin</i> expression on pSC101 ori vector	Hughes KT, et al., Phase variation in <i>Salmonella</i> : analysis of <i>Hin</i> recombinase and <i>hix</i> Recombination site interaction in vivo. <i>Genes Dev.</i> 1988 Aug;2(8):937-48.
pAT295	expresses Tn1545 excision enzymes <i>int</i> and <i>xis</i> on pHSG576 vector under <i>lac</i> control	Poyart-Salmeron C, et al. Molecular characterization of two proteins involved in the excision of the conjugative transposon Tn1545: homologies with other site-specific recombinases. <i>EMBO J.</i> 1989 Aug;8(8):2425-33.
pCP20	contains <i>Flp</i> cloned under control of temperature sensitive <i>lambda</i> repressor pR on a temperature sensitive plasmid derived from pHSG415	Cherepanov PP, Wackernagel W. Gene disruption in <i>Escherichia coli</i> : TcR and KmR cassettes with the option of <i>Flp</i> -catalyzed excision of the antibiotic-resistance determinant. <i>Gene.</i> 1995 May 26;158(1):9-14).
pEAW38	bearing the <i>Flp</i> gene under control of <i>lambda</i> pR and temperature sensitive repressor on pACYC vector.	Huang LC, et al., A bacterial model system for chromosomal targeting. <i>Nucleic Acids Res.</i> 1991 Feb 11;19(3):443-8).
pHSG		Hashimoto-Gotoh T, Franklin FC, Nordheim A, Timmis KN. Specific-purpose plasmid cloning vectors. I. Low copy number, temperature-sensitive, mobilization-defective pSC101-derived containment vectors. <i>Gene.</i> 1981 Dec;16(1-3):227-35.



[30] All references cited herein are incorporated by reference in their entirety for all purposes.

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[31] What is claimed is: